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PATENT- OCH REGISTRERINGSVERKET

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(21) *Patentansökningsnummer* *0202110-3*
*Patent application number*

(86) *Ingivningsdatum* *2002-07-05*
*Date of filing*



*Stockholm, 2003-07-10*

*För Patent- och registreringsverket*
*For the Patent- and Registration Office*

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*Avgift*
*Fee*

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## FIELD OF INVENTION

This invention relates to a composition comprising a mixture of at least two iscom complexes or iscom matrix complexes, each complex comprising one saponin fraction from *Quillaja Saponaria* Molina, and the use thereof as immunomodulators or adjuvants in formulations to be used for immunisations including vaccines. Especially the invention relates to the use of purified, semipurified or defined fractions of quillaja saponin in iscom and iscom-matrix adjuvanted vaccines. The use of saponin preparations according to this invention results in products with increased tolerability and increased immunogenicity. The preparations may be used in methods to tailor the immunogenicity with increased control of inflammatory, hypersensitivity and allergic reactions.

## PRIOR ART

The immune stimulatory properties of quillaja saponins have been known for long (Ramon 1926) and quillaja saponins have been used in free form, sometimes in combination with $Al(OH)_3$ in commercial vaccines since 1950:s (Dalsgaard 1978, Ma et al. 1994, Espinet 1951). A substantially more efficient use of the quillaja saponins compared to conventional free forms was described by Morein et al., in 1984 – the ISCOM technology (EP 0 109 942 B1, EP 0 242 380 B1 and EP 0 180 564 B1) and a few years later the ISCOM-matrix technology (Lövgren and Morein 1988, EP 0 436 620 B1). Using the iscom technology vaccine antigens are incorporated into a 40 nm complex consisting of quillaja saponins, cholesterol and phospholipid(s). The ISCOM-matrix technology employs the quillaja saponin: cholesterol:phospholipid complex in mixture (not associated) with the antigen(s). Both technologies decreases or abolish the haemolytic activity of the quillaja saponins, a property casing local side effects and adds to the overall toxicity of quillaja saponin preparations (Bomfod et al 1992).

Quillaja saponin preparations are heterogeneous mixtures of surface-active glycosides and serious problems in finding/defining batches with predicted and consistent adjuvant activity led to the isolation and characterisation of a "homogenous" fraction denoted "*Quillaja Saponaria* Molina" (Dalsgaard, 1974). This fraction was later shown to contain a range of related structures that were further purified into fractions/peaks by means of reversed phase HPLC (Kensil 1988, 1991, Kersten 1990 EP 0 362 279 B2, EP 0 555 276 B1). The motivation for this purification was not only to produce homogenous fractions of saponins that were readily characterised and defined but also to define a less toxic product. Acute toxicity or side effects have been major concerns for both veterinary and particularly human use of quillaja saponins in vaccine preparations. Theses goals were only partially met with success, the purified fractions e.g., QA-21 (EP 0 362 279 B2) and combinations of fractions A and C (WO 96/11711, Iscotec-patent) were indeed chemically defined compared to "*Quillaja Saponaria* Molina" but they still caused some toxicity and side effects. Despite the fact that fraction A virtually lack toxicity, a mixture consisting of 70% fraction A and 30% fraction C was not or only marginally less toxic than 100 % of fraction C of *Quillaja Saponaria* Molina.

In work leading to the present invention it was also shown that the different quillaja saponin fractions had not only different toxicity but also different immunomodulating properties (Johansson et al., EP 0 362 279 B2). By combining these fractions different immunomodulating capacities were obtained e.g. a Th1 driving or a Th2 driving capacity. It is, however, desirable to reduce the side effects that limits the amount of each fraction to be used in a tolerable formulation.

## SUMMARY OF THE INVENTION

The present invention relates to the use of at least two purified peaks or defined fractions of quillaja saponin in iscom and iscom-matrix as separate entities (particles). I.e. these fractions are not combined in the very same iscom or iscom-matrix particles, and the particles with different loads are mixed together to

constitute a formulation for immunisation. It has surprisingly turned out that a mixture of iscom or iscom matrixes each comprising a different fraction of *Quillaja Saponaria* Molina has lower toxicity than when these *Quillaja Saponaria* Molina fractions are integrated into the same iscom or iscom matrix particle. For example the mixture of fraction A-matrix and fraction C-matrix, or the use of fraction A-matrix or fraction C-matrix alone were considerably less toxic in mice than when the same fractions were integrated in the same iscom matrix (Example 4, table 1). Further, the immunogenicity or immune modulating properties are easier to tailor, and the possibilities are considerably enhanced to make improved vaccine formulations optimised both for the target species and the needs/requirements of the vaccine antigens.

Mice are particularly sensitive to quillaja saponins and overdosing leads to death within 4 days, often within 24 hours. Therefor mice were used to monitor the effects of toxicity and immunogenicity of the formulations prepared according to this invention. The interspecies variation in sensitivity to quillaja saponin is huge and reflects the needs for species optimisation to obtain tolerable formulations, but also for steering to obtain optimal immunogenicity of vaccine formulations. E.g. equines do not die from large doses of quillaja saponin, but they are prone to develop fever and local side effects after injection with free *Quillaja Saponaria* Molina, iscoms and iscom-matrix produced from *Quillaja Saponaria* Molina or mixed fractions of *Quillaja Saponaria* Molina.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a composition comprising a mixture of at least two iscom complexes each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina. The iscom complex may be an iscom matrix complex or an iscom complex.

Iscom contains at least one glycoside, at least one lipid and at least one type of antigen substance. The lipid is at least a sterol such as cholesterol and optionally also phosphatidyl choline. This complexes may also contain one or more other immunomodulatory (adjuvant-active) substances, and may be produced as described in EP 0 109 942 B1, EP 0 242 380 B1 and EP 0 180 564 B1.

An iscom matrix, comprises at least one glycoside and at least one lipid. The lipid is at least a sterol such as cholesterol and optionally also phosphatidyl choline. The iscom complexes may also contain one or more other immunomodulatory (adjuvant-active) substances, not necessarily a saponin, and may be produced as described in EP 0 436 620 B1.

The composition according to the invention may comprise iscom or iscom matrix complexes only or mixtures of iscom complex and iscom matrix complex. Different iscom and/or iscom matrix may be mixed wherein different saponin fractions from *Quillaja Saponaria* Molina are used.

The invention also covers the use of a mixture of at least two iscom or iscom matrix complexes each comprising one saponin fraction from *Quillaja Saponaria* Molina for the preparation of an immunomodulating pharmaceutical.

Another aspect of the invention is the use of a mixture of at least two iscom or iscom matrix complexes according to claim 1 each comprising one saponin fraction from *Quillaja Saponaria* Molina and at least one antigen for the preparation of a vaccine.

A further aspect of the invention is the use of a mixture of at least two iscom matrix complexes according to claim 1 each comprising one saponin fraction from *Quillaja Saponaria* Molina for the preparation of an adjuvant.

The immunogen which is incorporated into or associated with the iscom matrix in accordance with this invention may be any chemical entity which can induce an immune response in an individual such as (but not limited to) a human or other animal, including but not limited to a humoral and/or cell-mediated immune response to bacteria, viruses, mycoplasma or other micro-organisms. The specific immunogen can be a protein or peptide, a carbohydrate, polysaccharide, a lipo-polysaccharide or a lipopeptide; or it can be a combination of any of these.

Particularly, the specific immunogen can include a native protein or protein fragment, or a synthetic protein or protein fragment or peptide; it can include glycoprotein, glycopeptide, lipoprotein, lipopeptide, nucleoprotein, nucleopeptide; it can include a peptide-peptide conjugate; it can include a recombinant nucleic acid expression product.

Examples of such immunogens are cited in EP 0 109 942 B1 and include, but are not limited to, those that are capable of eliciting an immune response against viral or bacterial hepatitis, influenza, diphtheria, tetanus, pertussis, measles, mumps, rubella, polio, pneumococcus, herpes, respiratory syncytial virus, haemophilias influenza, chlamydia, varicella-zoster virus, rabies or human immunodeficiency virus.

The antigens may be incorporated into iscom or coupled on to iscom or iscom matrix or mixed with iscom and/or iscom matrix. Any mixtures of such iscom or iscom matrix may be used. One or more antigens may be used and a transport and passenger antigen may be used as described in EP 9600647-3 (PCT/SE97/00289).

The lipids used are particularly those described in the applicant's patent EP 0 109 942 B1 in particular on p. 3 and in patent EP 0 436 620 B1 on p. 7 lines 7-24. Especially sterols such as cholesterol and phospholipids such as phosphatidyl-ethanolamin and phosphatidylcolin are used. Lipid-containing receptors that bind to the cell-binding components, such as glycolipids including the cholera toxin's

receptor, which is the ganglioside GM1, and fucosed blood group antigen may be used. The cell-binding components can then function as mucus targeting molecule and be bound to the lipid-containing substances through simply mixing them with complexes that contain them. Iscom complexes comprising such receptors and receptors are described in WO 97/30728

The term " one saponin fraction from *Quillaja Saponaria* Molina." is used throughout this specification and in the claims as a generic description of a semi-purified or defined saponin fraction of *Quillaja Saponaria* or a substantially pure fraction. It is important that the fraction does not contain as much of any other fraction to negatively affect the good results that are obtained when the mixtures of iscom or iscom matrix comprising essentially one fraction is used. The saponin preparation may, if desired, include minor amounts for example up to 40% by weight, such as up to 30 % by weight, up to 25 % by weight, up to 20 % by weight, up to 15 % by weight, up to 10 % by weight, up to 7 % by weight, up to 5 % by weight, up to 2 % by weight, up to 1 % by weight, up to 0,5 % by weight up to 0,1 % by weight of other compounds such as other saponins or other adjuvant materials.

The saponin fractions according to the invention may be the A, B and C fractions described in WO 96/11711, the B3, B4 and B4b fractions described in EP 0 436 620 The fractions QA1-22 described in EP 0 3632 279 B2, Q-VAC (Nor-Feed, AS Denmark), *Quillaja Saponaria* Molina Spikoside (Isconova AB, Ultunaallén 2B, 756 51 Uppsala, Sweden )

The fractions QA-1-2-3-4-5-6-7-8-9-10-11-12-13-14-15-16-17-18-19-20-21 and 22 of EP 0 3632 279 B2, Especially QA-7, 17-18 and 21 may be used. They are obtained as described in EP 0 3632 279 B2, especially at page 6 and in Example 1 on page 8 and 9.

Fractions A, B and C described in WO 96/11711 are prepared from the lipophilic fraction obtained on chromatographic separation of the crude aqueous *Quillaja*

*Saponaria* Molina extract and elution with 70% acetonitrile in water to recover the lipophilic fraction. This lipophilic fraction is then separated by semipreparative HPLC with elution using a gradient of from 25% to 60% acetonitrile in acidic water. The fraction referred to herein as "Fraction A" or "QH-A" is, or corresponds to, the fraction, which is eluted at approximately 39% acetonitrile. The fraction referred to herein as "Fraction B" or "QH-B" is, or corresponds to, the fraction, which is eluted at approximately 47% acetonitrile. The fraction referred to herein as "Fraction C" or "QH-C" is, or corresponds to, the fraction, which is eluated at approximately 49% acetonitrile.

By combining iscom or iscom-matrix complexes comprising different fractions of *Quillaja Saponaria* Molina it is possible to produce preparations that are less toxic. It has also turned out that the effect of the compositions seems to be receptor mediated i.e. to receptors on the antigen presenting cells (APC) recognising the complexes. Thus, when two different fractions of *Quillaja Saponaria* Molina are integrated in the same iscom complex this complex may bind to receptors with affinity to fraction 1 plus receptors with affinity to fraction 2, i.e. two sets of receptors. Whereas when the fractions are in separate iscom particles or iscom matrix particles each particle will bind to the corresponding receptor(s) and limited to the receptors for which it has affinity. When two sets of receptors on the APC are triggered by the same particle that may cause strong effects leading to side effects. Moreover, the way that the complexes effect their action via receptors may be different in different species. Therefore, any combination of weight % of iscom complexes based on their content of different fractions of *Quillaja Saponaria* Molina may be used.

The use of saponin preparations according to this invention results in products with increased tolerability, increased immunogenicity. The preparations may be used in methods to tailor the immunogenicity including increased control of inflammatory, hypersensitivity and allergic reactions. This tailor making may be species dependent and may affect toxicity, tolerability and immunogenicity.

Any ratio of subfragments of *Quillaja Saponaria* Molina saponins may be used. Also, any combination of subfragments of *Quillaja Saponaria* Molina may be used. Thus, two or more subfragments may each be integrated into iscom or iscom matrix complex and used in the mixture according to the invention.

Preferably mixtures of iscom and /or matrix are used in which the fraction *Quillaja Saponaria* Molina and fraction Quil C are separately incorporated into different iscom complexes or matrix. As mentioned above any combinations of weight % of the different iscom complexes based on their content of fraction A and C of *Quillaja Saponaria* Molina respectively may be used. The mixtures may comprise from 5 to 95% by weight, 10 to 90% by weight 15 to 85% by weight, 20 to 80% by weight, 25 to 75% by weight, 30 to 70% by weight, 35 to 65% by weight, 40 to 60% by weight, 45 to 55% by weight, 40 to 60%, by weight, 50 to 50% by weight, 55 to 45% by weight, 60 to 40% by weight, 65 to 35% by weight, 70 to 30% by weight, 75 to 25% by weight, 80 to 20% by weight, 85 to 15% by weight, 90 to 10% by weight, 95 to 05% by weight, of iscom complexes comprising fraction A of *Quillaja Saponaria* Molina (as herein defined) and the rest up to 100 % in each case of interval of iscom complexes comprising fraction C of *Quillaja Saponaria* Molina (as herein defined), counted on the content of fraction A and C of *Quillaja Saponaria* Molina respectively in the iscom complexes.

Preferably, the mixture comprises from 90% to 99% by weight of complexes of fraction A and 1% to 10% by weight of complexes of fraction C. A particularly preferred preparation comprises about 91% to 98% by weight of complexes of fraction A and about 2% to 9% by weight of complexes of fraction C, especially about 92% to 96% by weight of complexes of fraction A and about 4% to 8% by weight of complexes of fraction C.

All intervals mentioned above may be used for any combination of any fraction of *Quillaja Saponaria* Molina in formulations for administration to any type of human

or animal species. Examples of animal species to which the formulations according to the invention may be administrated are companion animals such as cats, dogs, horses, birds such as parrots, economical important species such as cattle, e.g. bovine species, swines, sheep, goats. For horses especially combinations of fractions A and C may be used in any of the above mentioned ratios. Preferably more than 50% by weight of fraction C is used in combination with any of the other fractions and especially in combination with fraction A. Thus, from 50.5 – 99.5% by weight of C and 0,5 - .49,5% by weight of A may be used.

When prepared as described herein, Fractions A, B and C of *Quillaja Saponaria* Molina each represent groups or families of chemically closely related molecules with definable properties. The chromatographic conditions under which they are obtained are such that the batch-to-batch reproducibility in terms of elution profile and biological activity is highly consistent.

The present invention also extends to a vaccine composition comprising as the active component thereof either (i) an immunogenic iscom as broadly described above or (ii) an iscom matrix as broadly described above and at least one immunogen, together with one or more pharmaceutically acceptable carrier and/ or diluents.

The formulation of such vaccine compositions is well known to persons skilled in this field. Suitable pharmaceutically acceptable carriers and/or diluents include any and all conventional solvents, dispersion media, fillers, solid carriers, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art, and it is described, by way of example, in Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Company, Pennsylvania, USA. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the pharmaceutical

compositions of the present invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The iscom or iscom matrix complex according to the invention comprising each essentially one fraction of *Quillaja Saponaria* Molina may be administrated as a mixture or separately at the same administration site or at different administration sites at the same or at different times. Different fractions of *Quillaja Saponaria* Molina may be used in the different iscom complexes and matrix complexes and in the different compositions.

The invention therefore also relates to a kit of parts comprising at least two parts, wherein each part comprises one iscom complex or one iscom matrix complex each complex comprising one saponin fraction from *Quillaja Saponaria* Molina. Different fractions of *Quillaja Saponaria* Molina may be used in the different iscom complexes and matrix complexes in the different compositions in the different parts.

The compositions and kit of parts according to the invention may also comprise at least one other adjuvant than fractions from *Quillaja Saponaria* Molina. These adjuvants may be mixed with the iscom and/or iscom matrix complexes or be integrated into the complexes.

Examples of other adjuvants that can be incorporated in the iscom and iscom matrix are any adjuvant, natural or synthetic, with desired imunomodulatory effect, e.g. muramyl dipeptide (MDP)-derivatives, such as fatty acid, substituted MDP, threonyl analogues of MDP; DDA, poly anions such as Dextran sulphate, lipopolysaccarides such as saponins (other than Quil A). ("Future prospects for vaccine adjuvants", Warren, H.S. (1988) CRC Crit. Rev. Immunol. 8:2, 83-101; "Characterisation of a non-toxic monophosphoryl lipid A", (1987) Johnson, A.G. et al, Rev. Infect. Dis. 9:5, 5512-5516; "Developmental status of synthetic immunomodulators", Berendt, M.J. et al (1985), Year Immunol. 193-201; "Immunopotentiating conjugates", Stewart-Tull, D.E., Vaccine, 85, 3:1, 40-44).

It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the human subjects to be treated; each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier and/or diluent.

In yet another aspect, the present invention extends to a method of eliciting or inducing an immune response in an individual, which comprises administering to the individual an immunologically effective amount of a vaccine composition as broadly described above.

As previously mentioned, the individual may be a human or other animal, including a livestock animal (e.g. sheep, cow or horse), laboratory test animal (e.g. mouse, rat, rabbit or guinea pig), companion animal (e.g. dog or cat) or wild animal.

An immunologically effective amount means that amount necessary at least partly to attain the desired immune response, or to delay the onset of, inhibit the progression of, or halt altogether, the onset or progression of the particular condition being treated. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated, the capacity of the individual's immune system to synthesise antibodies, the degree of protection desired the formulation of the vaccine, the assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated component or

group of components but not the exclusion of any other components or group of components.

The invention is illustrated by the following figures of which:

Figure 1 shows the preparation of fractions A, B and C by HPLC;

Fig 2 shows antigen specific antibody responses against influenza virus micelles as described in the text were tested in ELISA (log Titre) in the IgG1 ((A) and IgG2a (B) subclasses. Mice (female NMRI) were immunised weeks 0 and 4 with the vaccine formulations described in Table 2 i.e. groups 1 through 8. Mice were bled weeks 3 and 6. The antibody responses were tested from bleeding collected at week 6.

Fig 3 shows the cell mediated immune response measured as the production of the cytokines IL-5 (A) and IFN-γ (B) by spleen cells collected week 6 after immunisation as described in Fig 2 after stimulation in vitro with influenza virus micelles as described in the text.

Figure 4 shows serological response against different equine influenza antigens, 2 weeks after the second vaccination. Adjuvants 1 = Quil A, 2 = A, 3 = C, 4 = F, 5 = G and 6 = neg control.

All publication mentioned herein are hereby incorporated as reference. The invention will now be described by the following non-limiting examples.

EXAMPLE 1 Preparation of *Quillaja Saponaria* Molina subfragment saponins.

Purification of crude *Quillaja Saponaria* Molina extract to fractions A, B and C.
A solution (0.5ml) of crude Quillaja bark extract in water (0.5 g/ml) is pre-treated on a sep-pak column (Waters Associates, MA).

The pre-treatment involves washing of the loaded sep-pak column with 10% acetonitrile in acidic water in order to remove hydrophilic substances. Lipophilic substances including QH-A, QH-B and QH-C are then eluted by 70% acetonitrile in water.

The lipophilic fraction from the sep-pak column is then separated by a semipreparative HPLC column (CT-sil, C8, 10 X 250mm, ChromTech, Sweden). The sample is eluted through the column by a gradient from 25% to 60% acetonitrile in acidic water. Three fractions are collected from the HPLC column during the separation. The residues after evaporation of these three fractions constitute QH-A, QH-B and QH-C.

The fractions designated QH-A, QH-B and QH-C were eluted at approximately 39, 47 and 49% acetonitrile respectively. The exact elution profile and conditions are shown in Figure 1.

EXAMPLE 2. Prepartion of iscom matrix.

Materials

Cholesterol, e.g., Sigma C 8503)

Phosphatidyl choline (egg derived) e.g., Sigma P 3556

MEGA-10 (Bachem AG, Switzerland)

Quillaja saponin fractions A and C (patent WO9611711)

0,22 µm Sterile filters (Acrodisc)

PBS (10 mM phosphate buffered 150 mM saline, pH 6,8-7,4)

Slide-A-Lyzer casettes MW cut off 12-14.000 (Pierce)

MEGA-10 (stock solution)

Make a 20% (w/w) stock solution by adding 8 ml distilled water to 2.0 g of dry solid MEGA-10. Dissolve by gentle heating (30-50°C). Filter through a 0,22 nm sterile filter, aliquot and store at –20°C.

Lipid mixture (15 mg/ml)

Dissolve 100 mg of each cholesterol and phosphatidyl choline in 10 ml 20% MEGA-10. The lipids dissolve slowly at 30-60°C with slow stirring. Filter through a 0,22 nm sterile filter, aliquot and store at –20°C. After freezing, the lipid mixtures need to be heated up to 40 °C until clear. Temperate all solutions to 24±1 °C.

Saponin stock solutions (100 mg/ml)

1,0 gram of *Quillaja Saponaria* Molina fractions (A or C is dissolved in sterile distilled water. Keep aliquots frozen at -20°C.). Filter through a 0,22 nm sterile filter, aliquot and store at –20°C.

The different iscom-matrix is preparations are produced as outlined in Table 1. Prepare the mixtures as follows.

Add 2 ml PBS to a 50 ml falcon tube

1 Add the lipid mixture and mix thoroughly
2 Add saponin and mix thoroughly
3 Add PBS to a final volume of 12,0 ml, mix thoroughly
4 Incubate for 30 minutes
5 Fill into Slide-A-lyzer
6 Dialyse against 4 changes of 2 liters of PBS (24±1°C) (for 48-60 hours)
7 Aspirate from Slide-A-Lyser and filter through 0,22 nm sterile filter.

The formation of iscom-matrix was verified by negative staining electron microscopy and the resulting concentrations of quillaja saponin was determined by HPLC.

Table 1

| Preparation | Lipid-mixture | | Quillaja saponin | | PBS |
|---|---|---|---|---|---|
| | Amount (mg) | Volume (µl) | Amount (mg) | Volume (µl) | Volume (ml) |
| A-matrix | 12 | 800 | 48 | 480 | 2,0 + 8,72 |
| C-matrix | 12 | 800 | 30 | 300 | 2,0 + 8,90 |

| | | | | | |
|---|---|---|---|---|---|
| 703-matrix | 12 | 800 | 42 | 420 | 2,0 +8,78 |

EXAMPLE 3. Preparation of PR-8 protein micelles.

1 Dilute 12 mg of PR-8 monomers (1,5 mg/ml) with PBS to a final protein concentration of 1,0 mg/ml.
2 Filter through 0,22 nm sterile filter
3 Fill into Slide-A-lyzer
4 Dialyse against 4 changes of 2 liters of PBS (24±1°C) (for 48-60 hours)
5 Aspirate from Slide-A-Lyser

EXAMPLE 4. Immunisation study.

This example was carried out to show in a comparative study that iscom-matrix composed of a mixture matrix particles provokes minimal degree of side effects. One set of particles contain QHA as the only saponin and the other set of particles contain QHC as the only saponin and were prepared according to Example 2. This formulation is named *matrix with a "mixture of particles"*. The comparison is done with an iscom matrix as described in Paten WO 96/11711 i.e. each particle contain both QHA and QHC in e.g. a ratio of 70% QHA and 30% of QHC. This is a *matrix with all in one particle.*

Mice were immunised days 0 and 42 with 1 µg of PR8 micelles (prepared as described in example 3) mixed with the iscom matrix formulation *matrix with a mixture of particles* and compared with the iscom *matrix with all in one particle*, or with iscom matrix containig 100% QHA or 100% QHC as described in Table 2. Groups in which more than 50% of the mice died or suffered from unacceptable side effects by the treatment were culled and excluded from further investigations.

Serum samples were taken from all mice in groups 1-7 day 56, two weeks after the booster administration. The sera were screened for antigen specific antibodies of

IgG1 (A) and IgG2a (B) subclasses. Group 8 in the figure represents unvaccinated mice. The results are shown in Fig 2.

After the second bleeding spleens were taken from two mice per group (groups 2, 4, 5, 6 and 7). The spleen cells were stimulated with PR8 micelles *n vitro* and the antigen specific induction of IL-5 (A) and IFN-γ (B) was measured. Group 8 in the figure represents unvaccinated mice. The results are shown in Fig 3.

Table 2

Mice were immunised days 0 and 42 with the iscom matrix formulation *matrix with a mixture particles* (MIX groups 1,2 and 3) and compared with, the iscom *matrix with all in one particle* (CONV groups 7, 8, 9, 10 and 11), or with iscom matrix containing 100% QHA (groups 4 and 5) or 100% QHC (group 6 and 12).

| Group no | Amount (μg) | A:C (ratio) | MIX/ CONV | No of mice | Dead#/total |
|---|---|---|---|---|---|
| 1 | 50 | 80:20 | MIX | 8 | 2/8 |
| 2 | 50 | 92:8 | MIX | 8 | 0/8 |
| 3 | 50 | 96:4 | MIX | 8 | 0/8 |
| 4 | 50 | 100% A | | 8 | 0/8 |
| 5 | 10 | 100% A | | 8 | 0/8 |
| 6 | 10 | 100% C | | 8 | 0/8 |
| 7 | 10 | 70:30 | CONV | 8 | 0/8 |
| 8 | 50 | 80:20 | CONV | 8 | 8/8 |
| 9 | 50 | 92:8 | CONV | 8 | 6/8 |
| 10 | 50 | 96:4 | CONV | 8 | 5/8 |
| 11 | 50 | 70:30 | CONV | 8 | 8/8 |
| 12 | 50 | 100% C | | 8 | 8/8 |
| | | | | | |

#Mice that were eutanized or died within 24 h after administration.

A:C ratio (weight) of quillaja saponin fraction A and C

RESULTS

Mice immunised PR8 micelles adjuvanted with high dose (50μg) of iscom matrix containing 80% QHA and 20% of QHC i.e. 10μg died within 1 or 2 days. Likewise did mice immunised with 50μg of the formulation 100% QHC died within 2 days. In contrast mice immunised with 50μg of the formulation 100% QHA survive without any noticed adverse reactions. I.e. the lower dose of QHC was sufficient to kill mice when incorporated in the same matrix particle as QHA (CONV group 8 in Table 2). Even when a low amount as 4μg (8(%) of QHC in combination with 46 μg (92%) of QHA in a CONV matrix killed 6 out of 8 mice (group 9 Table 2). Also 2μg (4%) QHC killed mice when combined with QHA in CONV matrix (group 10 Table 2). Mice in group 6 (Table 2) received 10μg QHC being 100% (i.e. no QHA) in the matrix and all mice survived.

Thus, the mice were more sensitive to QHC when combined with QHA in the same CONV matrix particle (groups, 8 9, and 10).

Mice receiving low dose matrix i.e. 10μg of total saponin divided on 70% of QHA and 30% of QHC survived all. In this case the mice received 3μg QHC.

Mice immunised with PR8 micelles adjuvanted with a formulation containing different matrix particles i.e. a mixture of particles (MIX) including one set of QHA and one set of QHC survived much higher doses of this matrix formulation than the CONV formulation. Mice injected with the formulation with 92% QHA (46μg) and 8% QHC (4μg) group 2 in Table 2 (92:8) or with the formulation 96:4 (Group 3 in Table 2) containing 2μg of QHC survived all. This outcome shall be compared with that of corresponding amounts of QHA and QHC in CONV matrix (groups 9 and 10 Table 2) which caused high mortality.

Thus, mortality – toxicity can be avoided by physical separation of QHC from QHA and distribute them into different matrix particles.

Enhancement of antibody response

The results are shown in figure 2. Antigen specific response divided into IgG subclasses. Mice (female NMRI) were immunised weeks 0 and 4 with the vaccine formulations described in Table 2. Mice were bled weeks 3 and 6. The IgG1 ($^{10}$ log Elisa titres) response at week 6 is shown in A and the corresponding IgG2 response in B.

An important finding in this experiment is that the immune enhancing capacity is retained or enhanced as measured by antibody responses when QHA and QHC are separated into different sets of particles, as demonstrated in Fig 2.

In Fig 2 it is shown that a mixture of particles (MIX) enhance the same level of IgG1 antibody (Fig 2A) to PR8 micelles as the same dose of QHA and QHC in the same proportions when incorporated in the same particle i.e. CONV particles. However, higher levels of IgG2a antibody antibodies were enhanced by the MIX formulation (Fig 2B). The groups 2 and 3 (MIX) shall be compared with the groups 9 and 10 (CONV) QHA –QHC matrix and with 100% of low dose QHC matrix (group 6) and with 100% of QHA high dose matrix (group 4 Table 2). Mice in group 7 injected with a low dose of 10µg (CONV 70:30), i.e. the dose the mice can accept, responded with a potent IgG1 (Fig 2A) response, but a low IgG2a response (Fig 2B).

Thus, the invention with a matrix formulation with a mixture of matrix particles can be given in high doses evading side effects, enhance the antibody response to higher levels than those with than the CONV matrix. Particularly the IgG2a response is enhanced. The IgG2a response is e.g. particularly important for defence against intracellular parasites e.g. viruses.

Enhancement of cell mediated immune response

The CONV matrix formulations have inferior capacity to enhance cell mediated immunity in the doses tolerated than the MIX formulations (Figs 3 A and B). The MIX formulation (92:8, group 2) enhance considerably higher IL-5 levels than the CONV (70:30.group 7). QHA-QHC formulation, or the 100% QHC matrix. Formulation (group 6). The mix (92:8, group 9) formulation also enhances the IFN-γ considerably better than the QHC 100% matrix (group 6) or the CONV (70:30, group 7) formulation.

To note is that QHA has a strong capacity to enhance cell mediated immune responses measured by IL-5 and IFN-γ production, but a low capacity to enhance antibody response.

In conclusion the invention defines a concept for iscom and iscom matrix formulations that considerably reduce toxicity and side effects allowing potent doses of the adjuvant active molecules without loosing capacity to enhance immune response.

Moreover, while a low but acceptable dose of a QHC matrix formulation has good capacity to enhance and IgG1 response it is lower with regard to the important IgG2a response. The capacity of QHC matrix to induce cell mediated immunity is also comparatively low to that of the invention.

The QHA matrix potently enhances cell mediated immunity, but is inferior to the invention to enhance antibody mediated immunity.

The invention with mixed matrix particles is superior to matrix formulations containing QHA and QHC in the same particle (CONV) measured by IgG2a antibody response and measured by cell mediated responses.

The new invention enhances a complete immune response and is therefore superior to the earlier described matrix formulations, which this example 4 shows.

EXAMPLE 5. Safety testing in horses of five different formulations of iscom –matrix as adjuvant in a vaccine against influenza.

Materials:

Iscom-matrix A (A-matrix), B A+C (7:3)-matrix, C (C-matrix) and D (A+C (3:7)-matrix were prepared according to EXAMPLE 2 The Iscom-matrices A, B, C and D were blended into vaccines A, B, C and D by mixing per millilitre vaccine, 250 µg matrix with 40 LF tetanus toxoid and 100, 50, and 50 AU equine influenza antigens derived from A/equi-1/Prague/56, A/equi-2/Newmarket-1/93 and A/equi-2/Newmarket-2/93, respectively (Table 3). Buffer 3 was added ad 1 ml per dose. Influenza antigens were prepared according to EXAMPLE 3. Horses were of mixed breed and consisted of Netherlands Welsh Ponies, Netherlands Forest Ponies and Netherlands Riding Ponies.

Methods:

The safety test was carried out according to the PH. Eur., 0249, Batch safety testing ( 1998). In brief, horses were vaccinated intramuscularly with a double dose of vaccine (2 ml/ animal; 500 µg adjuvant/animal) and revaccinated with a single dose 14 days later (1ml/animal; 250 µg adjuvant). Vaccines were administered at the left side of the neck, each vaccination on a different spot or at the right side of the neck when the animals were used to test the safety of two preparations. In the latter case experiments were conducted in a consecutive order. After each administration, local reactions were scored by palpation of the injection site and systemic reactions were scored by measuring rectal temperatures and monitoring clinical appearance of the animal.

Table 3

| | Influenza antigens (AU/ml) | | | | | |
|---|---|---|---|---|---|---|
| Vaccine | Prague/56 | NM-1/93 | NM-2/93 | Adjuvant | Tetanus (Lf/ml) | Volume (ml) |
| A | 100 | 50 | 50 | A, 250µg | 40 | 1 |
| B | 100 | 50 | 50 | B, 250µg | 40 | 1 |
| C | 100 | 50 | 50 | C, 250µg | 40 | 1 |
| D | 100 | 50 | 50 | D, 250µg | 40 | 1 |

Results

Systemic reactions measured as body temperature.

Horse no. 28, vaccinated using adjuvant B gave a body temperature increase of 1.6°C, 6h after the first vaccination. The body temperature 6h after the first vaccination was 38.9°C. After the second vaccination hardly any increase in body temperature was observed. The other horse (no. 50) did not respond with an increase in body temperature after vaccination.

Horse no 49, vaccinated using adjuvant D gave a body temperature increase of 1.9°C, 6h after the second vaccination. The body temperature 6h after the second vaccination was 39.3°C. The other horse (no. 53) also gave an increase in body temperature 6h after the first and second vaccination of 1.3°C and 0.9°C, respectively. The body temperatures of this horse were 38.6 And 38.1, respectively. Furthermore, the vaccine that contains adjuvant D induced a systemic reaction in horse no 53, after the first and second vaccination the animal was listless, lethargic, slow (data shown in table 4).

Finally, horse no. 28, vaccinated using adjuvant E gave a body temperature increase of 1.7°C, 6h after the second vaccination. The body temperature 6h after the second vaccination was 39.6°C. The other horse no. 50 did not respond with an increase in body temperature after vaccination.

Local reaction in horses scored by palpation of the injection side

Animals were observed for a period of 28 days after the first vaccination. Data have been recorded for the first 2 days after the two vaccinations because no local

reactions were observed at the other time points. As can be seen from table 4, no big fluctuations in body temperature were seen when the adjuvants A and C were being used.

Table 4: Local reaction score.

| | | Time after first vaccination (500µg of adjuvant) | | | | Time after second vaccination (250µg of adjuvant) | | |
|---|---|---|---|---|---|---|---|---|
| Horse no. | Adjuvant | 0h | 6h | 24h | 48h | 6h | 24h | 48h |
| 49 | A | - | - | - | - | - | - | D, 2 |
| 53 | A | - | - | - | - | - | - | - |
| 28 | B | - | D, 1* F +1,6°C | - | Snotty nose- | - | - | - |
| 50 | B | - | - | - | - | - | - | - |
| 47 | C | - | - | - | - | - | - | - |
| 52 | C | - | - | - | - | - | - | - |
| 49 | D | - | - F +1,9°C | - | - | D, 1 F +2,0°C | | |
| 53 | D | - | A F +1,3°C | A | - | A, D, 2.5 F +0,9°C | | |

A = Listless, lethargic, slow D = Diffuse local reaction

DP = Diffuse painful local reaction DW = Diffuse local reaction spot feels warm

F=increase of body temperature

* = Diameter of local reaction in cm

As is evident from table 4 adjuvants A and C did not induce local reactions, adjuvant B and D did not induce severe local reactions.

EXAMPLE 6. Potency testing in horses of two candidate formulations of iscom-matrix as adjuvant in a vaccine against equine influenza; comparison with two new formulations (according to this invention).

Materials

Iscom-matrix A (A-matrix) and C (C-matrix) were prepared according to EXAMPLE 2.

Iscom-matrix F and G were prepared by mixing of iscom-matrix A and iscom-matrix-C to equal composition (amounts) as iscom-matrix preparations B (A+C (7:3)-matrix) and D (A+C (3:7)-matrix) but with Quil A fraction A and Quil A fraction C in different iscom-matrix complexes.

Methods

In this example, the adjuvants A, C, F and G (1 ml/animal; 250 µg adjuvant/animal) were tested for potency. Horses were vaccinated twice with a 4-week interval. The serological responses against the antigens Prague, Newmarket/1/93, Newmarket/2/93 and tetanus toxoid (formulated with different adjuvants) were measured 2 weeks after the second vaccination. As positive control, animals vaccinated with a traditional vaccine containing the same amount of antigens but with free Quil A as adjuvant (125 µg/dose), i.e. Equenza, were included in the experiment.

It was shown that the adjuvants A, C, F and G at a dose of 250 µg/ml were able to enhance the serological responses when compared to a traditional vaccine. The serological response against equine influenza antigens was similar for all new adjuvants investigated (A,C,F and G). The serological response for the tetanus toxoid was comparable for the adjuvants A, C and F but were higher for adjuvant G. It was concluded that adjuvants A, C, F and G were suitable as new generation adjuvants in horses based on the potency data.

Results

Results of equine influenza antigens are summarised in figure 4 and the results for tetanus toxoid antigen are summarised in table 6. It was shown that the adjuvants A, C, F and G at a dose of 250µl/ml were able to enhance the serological responses when compared to a traditional vaccine (adjuvant Quil A). The serological response

against equine influenza antigens was similar for all new adjuvants investigated (A, C, F and G). The serological response for the tetanus toxoid was comparable for the adjuvants A, C and F but were higher for adjuvant G.

Figure 4 shows the serological response against different equine influenza antigens, 2 weeks after the second vaccination. Adjuvants 1 = Quil A, 2 = A, 3 = C, 4 = F, 5 = G and 6 = neg control.

Table 5: Serological response against tetanus toxoid antigen, 2 weeks after the second vaccination.

| Group | Adjuvant | Amount of adjuvant | Serological response |
|---|---|---|---|
| 1 | Quil A (Intervet) | 125µg/ml | 4.7 |
| 2 | A | 250µg/ml | 9.6 |
| 3 | C | 250µg/ml | 11.2 |
| 4 | F | 250µg/ml | 6.8 |
| 5 | G | 250µg/ml | 21.2 |
| 6 | - | - | <0.4 |

EXAMPLE 7. Safety testing in horses of adjuvants A, C, F and G in a vaccine against equine influenza.

Materials

Iscom-matrix A (A-matrix) and C (C-matrix) were prepared according to EXAMPLE 2.

Iscom-matrix F and G were prepared by mixing of iscom-matrix A and iscom-matrix-C to equal composition (amounts) as iscom-matrix preparations B (A+C (7:3)-matrix) and D (A+C (3:7)-matrix) but with Quil A fraction A and Quil A fraction C in different iscom-matrix complexes.

Methods

In Example 7 preparations A, C, F and G were tested for safety in horses. The safety test was carried out according to the Ph. Eur., 0249, Batch (safety) testing (1998) as

described in example 3 with the exception that a double dose contained 1000 µg adjuvant/animal.

Results

No systemic reactions were observed in preparations containing adjuvant A, C and F. Preparation G induced systemic reactions in 4 out of 5 animals after the first and second vaccination (temperatures increased between 1,3 and 1,8 °C and were ≥ 38,7 °C). Regarding local reactions preparation F was most safe. However the local reactions induced by the other preparations were acceptable (small local reactions, soft, 1 x 1 cm to 3 x 3 cm at 4-6h after the first vaccination).

GENERAL CONCLUSION from examples 5, 6 and 7

In EXAMPLE 5 in which 250µg of adjuvat was used preparations B and D induced some local and systemic side effects. The adjuvant effect of adjuvant preparation B and D were equal to that of adjuvant preparation C. The same compositions as B and D but with the saponin fractions A and C in separate matrix complexes, i.e., preparations F and G were in Example 6 shown to be as potent as (or as in the case of adjuvant G even more potent) adjuvant preparation C. In Example 7 the safety of 1000 µg (twice as much as the primary injection in Example 5) of adjuvant preparations A, C, F and G were tested. Adjuvant preparations A, C and F did not induce severe local or systemic reactions. Adjuvant preparation G did induce systemic reactions (increased body temperature, 1,3-1,8 °C, 6 h post vaccination) in 4 of 5 animals

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## CLAIMS

1. A composition comprising a mixture of at least two iscom complexes, chosen from iscom and iscom matrix complexes, each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina.

2. A composition according to claim 1 comprising a mixture of at least two iscom complexes each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina.

3. A composition according to claim 1 comprising a mixture of at least two iscom matrix complexes each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina.

4. A composition according to claim 1 comprising a mixture of at least two iscom and/or iscom matrix complexes each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina, which fraction may be a different one in the different complexes.

5. Kit of parts comprising at least two parts, wherein each part comprises one iscom complex or one iscom matrix complex, each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina, which fraction may be a different one in the different complexes.

6. A composition according to any of claims 1-6 comprising at least one other adjuvant than a saponin fraction from *Quillaja Saponaria* Molina.

7. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-6 each comprising essentially one saponin fraction from *Quillaja Saponaria* Molina for the preparation of an immunomodulating pharmaceutical.

8. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-6 each comprising essentially one saponin fraction from *Quillaja Saponaria* Molina and at least one antigen for the preparation of a vaccine.

9. Use of a mixture of at least two iscom matrix complexes according to any of claims 3,5 and/or 6 each comprising essentially one saponin fraction from *Quillaja Saponaria* Molina for the preparation of an adjuvant.

10. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-6, characterised in that the saponin fraction from *Quillaja Saponaria* Molina is chosen from fraction A, fraction B, fraction C of *Quillaja Saponaria* Molina, spicoside, Q VAC, Quil 1-21

11. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-6, characterised in that the saponin fraction from *Quillaja Saponaria* Molina is chosen from fraction A of *Quillaja Saponaria* Molina, fraction B of *Quillaja Saponaria* Molina, and fraction C of *Quillaja Saponaria* Molina.

12. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-6, characterised in that the mixture comprises from 50% to 70% by weight of complexes of fraction A of *Quillaja Saponaria* Molina and from 50% to 30% by weight of complexes of fraction C of *Quillaja Saponaria* Molina.

13. Use of a mixture of at least two iscom or iscom matrix complexes according to a claim 9, characterised in that the mixture comprises from 50% to 30% by weight of complexes of fraction A of *Quillaja Saponaria* Molina and from 50% to 70% by weight of complexes of fraction C of *Quillaja Saponaria* Molina.

14. Use of a mixture of at least two iscom or iscom matrix complexes according to any of claims 1-3, characterised in that the saponin fraction from *Quillaja Saponaria* Molina is chosen from Quil 1-21.

## ABSTRACT

The invention relates to a composition comprising a mixture of at least two iscom complexes each complex comprising essentially one saponin fraction from *Quillaja Saponaria* Molina. The complexes may be iscom complexes or iscom matrix complexes. The invention also pertains to the use of such a mixture for the preparation of an immunomodulating pharmaceutical, and adjuvant, formulations for immunsation e.g. for production of monoclonal antibodies and a vaccine. Kits of parts comprising at least two parts, wherein each part comprises one iscom complex or one iscom matrix complex according to the invention are also embraced.

FIG 1

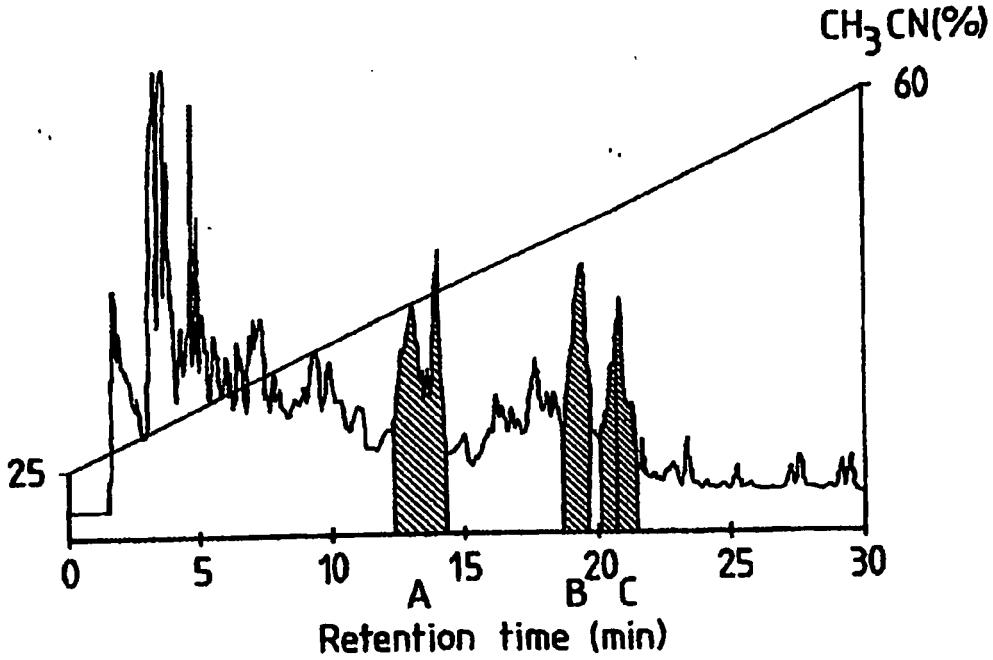
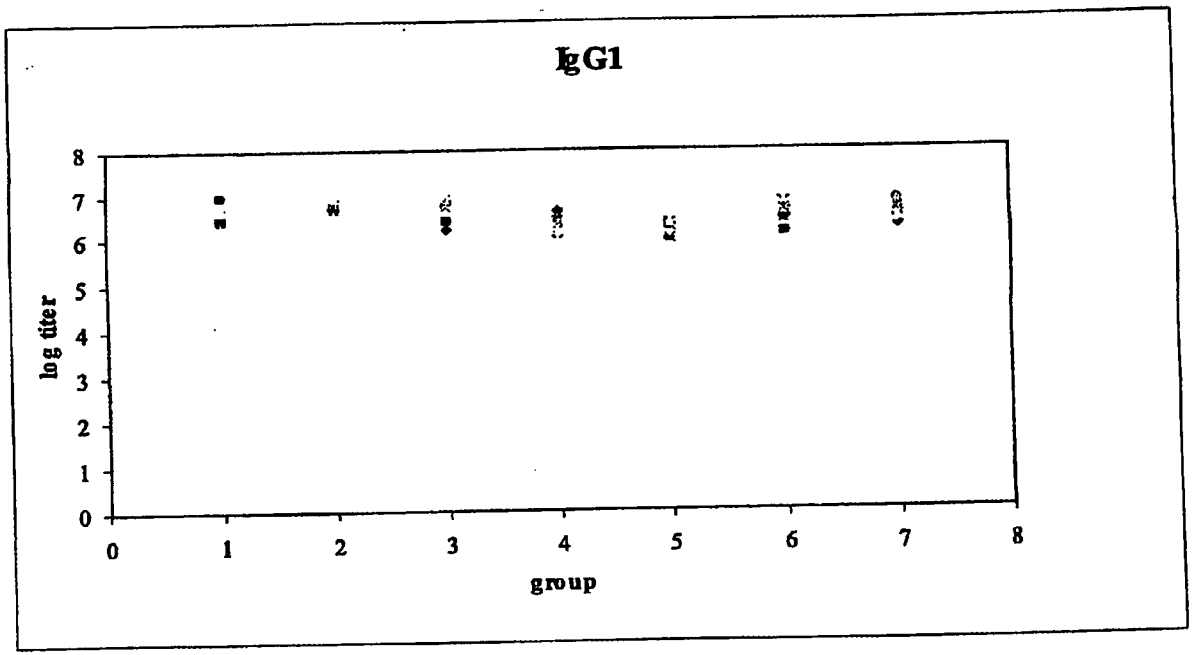


Fig. 2A



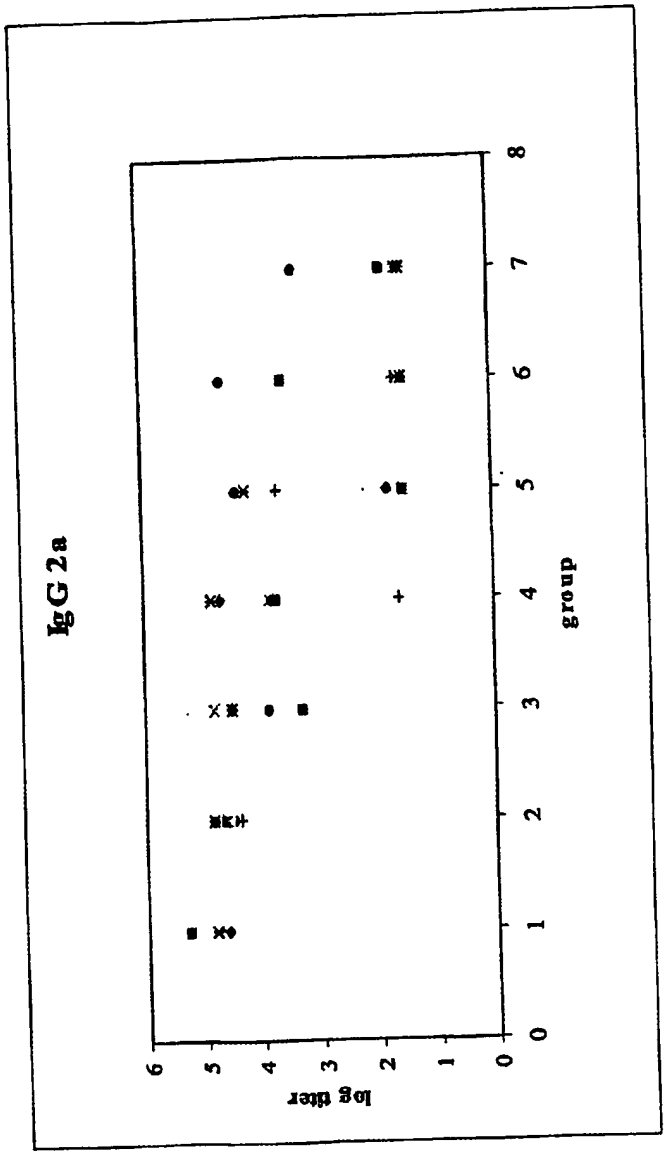
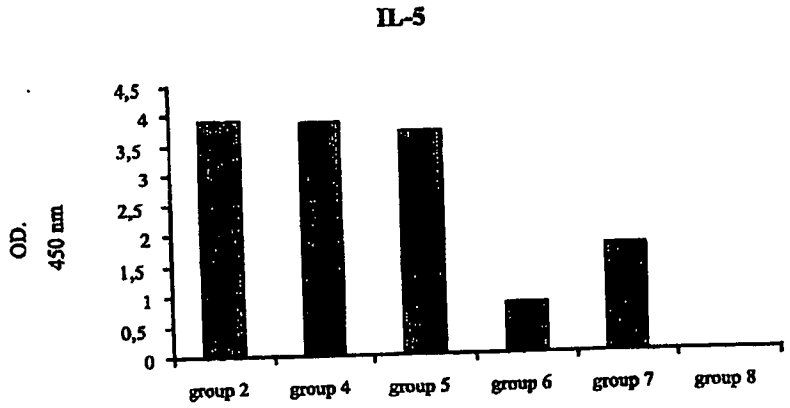



Fig. 2B

Fig. 3A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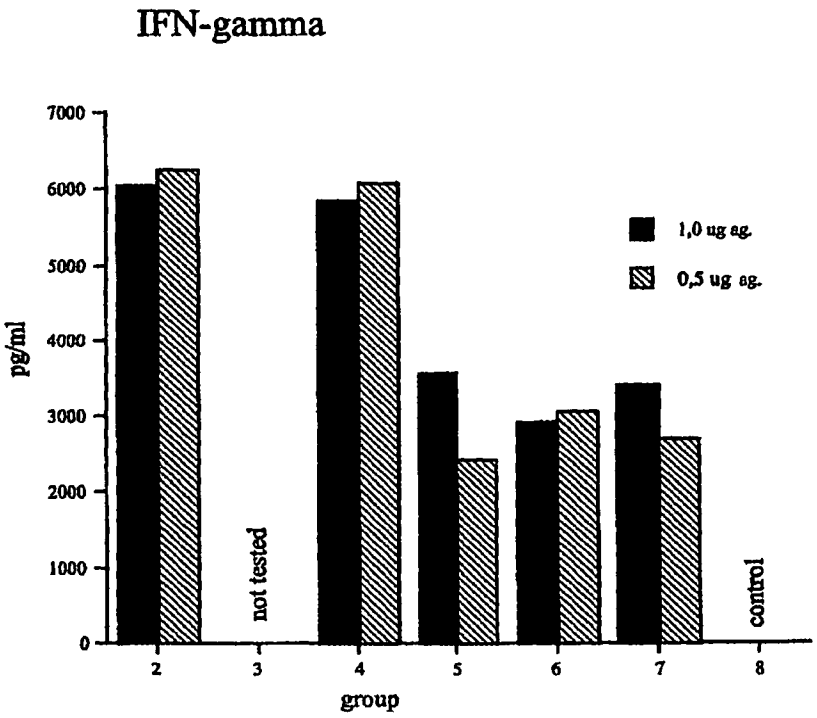
4
3,5
3
2,5
2
1,5
1
0,5
0
group 2
group 4
group 5
group 6
group 7
group 8

Fig. 3B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6000
5000
4000
3000
2000
1000
0
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1,0 ug ag.
0,5 ug ag.
not tested
control
2
3
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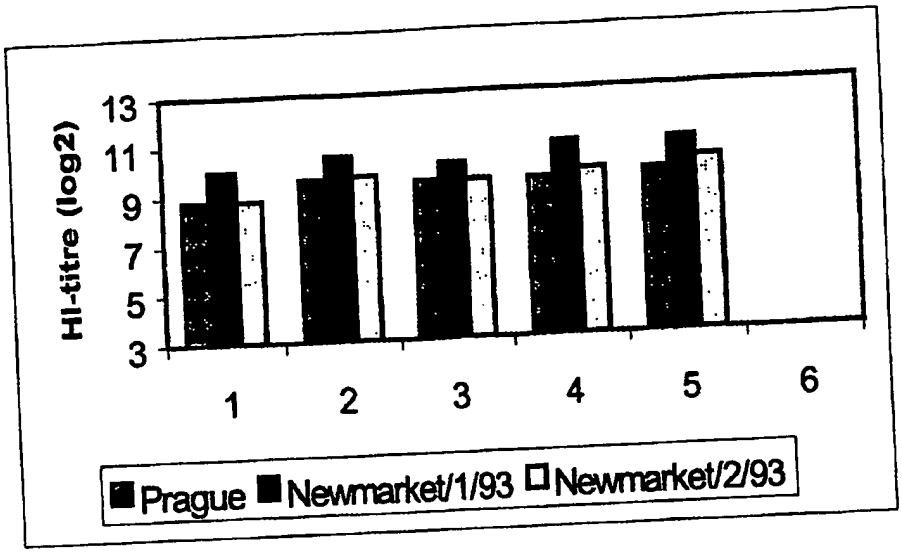
6
7
8
group

Fig. 4


HI-titre (log2)
13
11
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2
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Prague
Newmarket/1/93
Newmarket/2/93